The Use of Receiver Operating Characteristic (ROC) Curves as a Tool to Assess Noise in the Targeted Sequencing of Forensic Short Tandem Repeat (STR) Markers

Sarah Riman[1], PhD; Hari Iyer[2], PhD; Lisa Borsuk[1], MS;
Peter M. Vallone[1], PhD

[1]Applied Genetics Group, National Institute of Standards and Technology
[2]Statistical Design, Analysis, and Modeling Group

Overview

- Comparison of conventional CE versus NGS-STR genotyping workflows
- Comparison of conventional CE versus NGS-STR data analysis
- Scope of the work
- Discussion of a sensitivity study consisting of single-source DNA profiles generated by targeted sequencing

Comparison of conventional CE versus NGS-STR genotyping workflows

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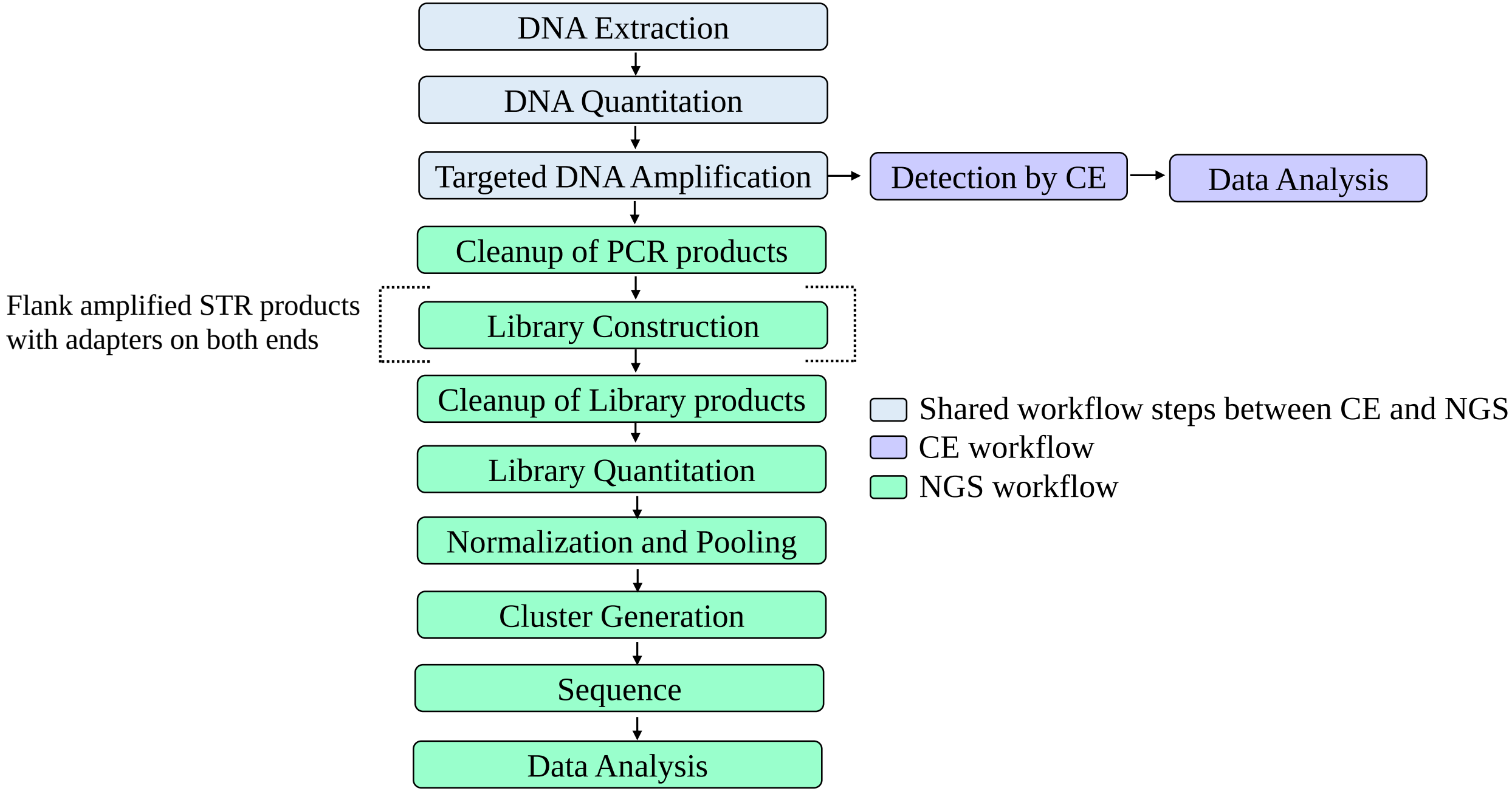
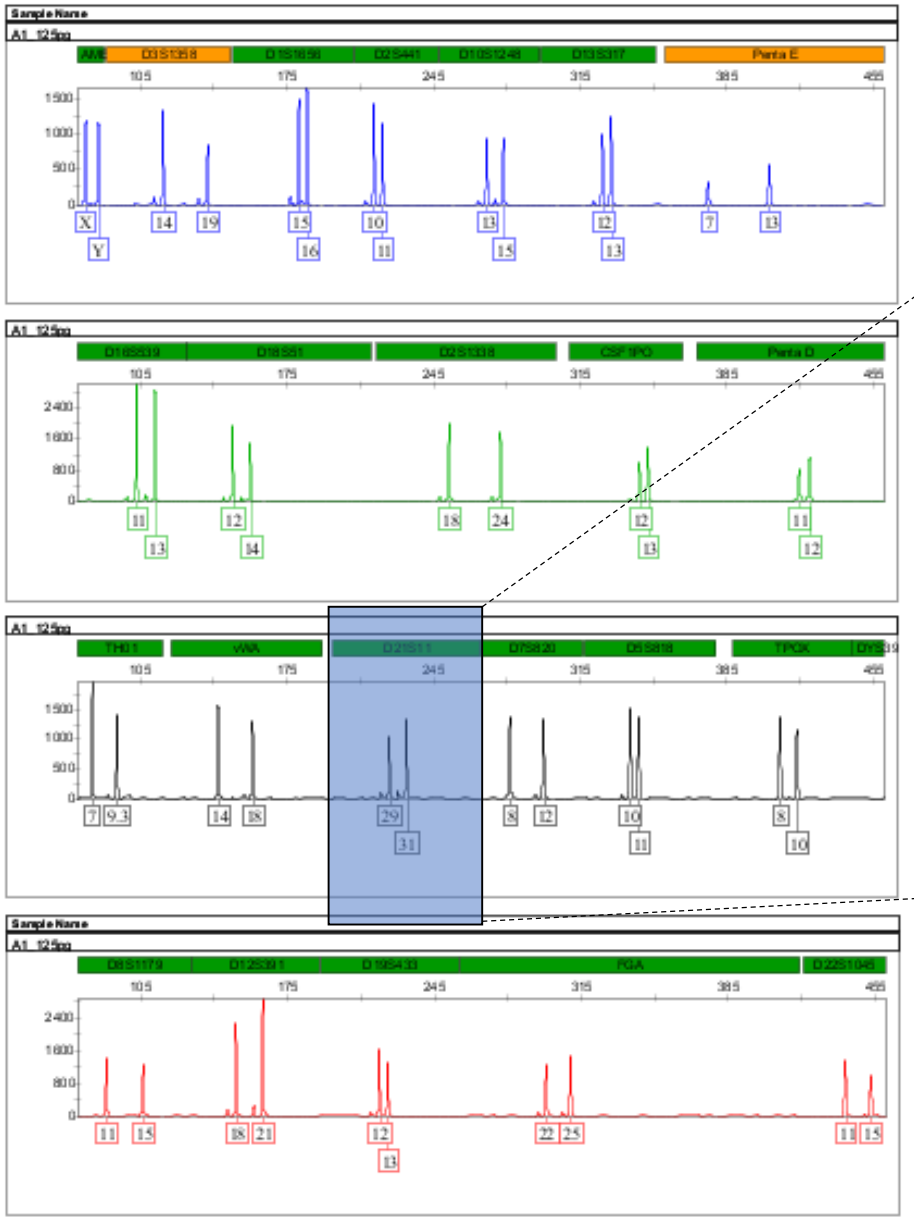


- Targeted sequencing of STR markers relies on the PCR-amplification process

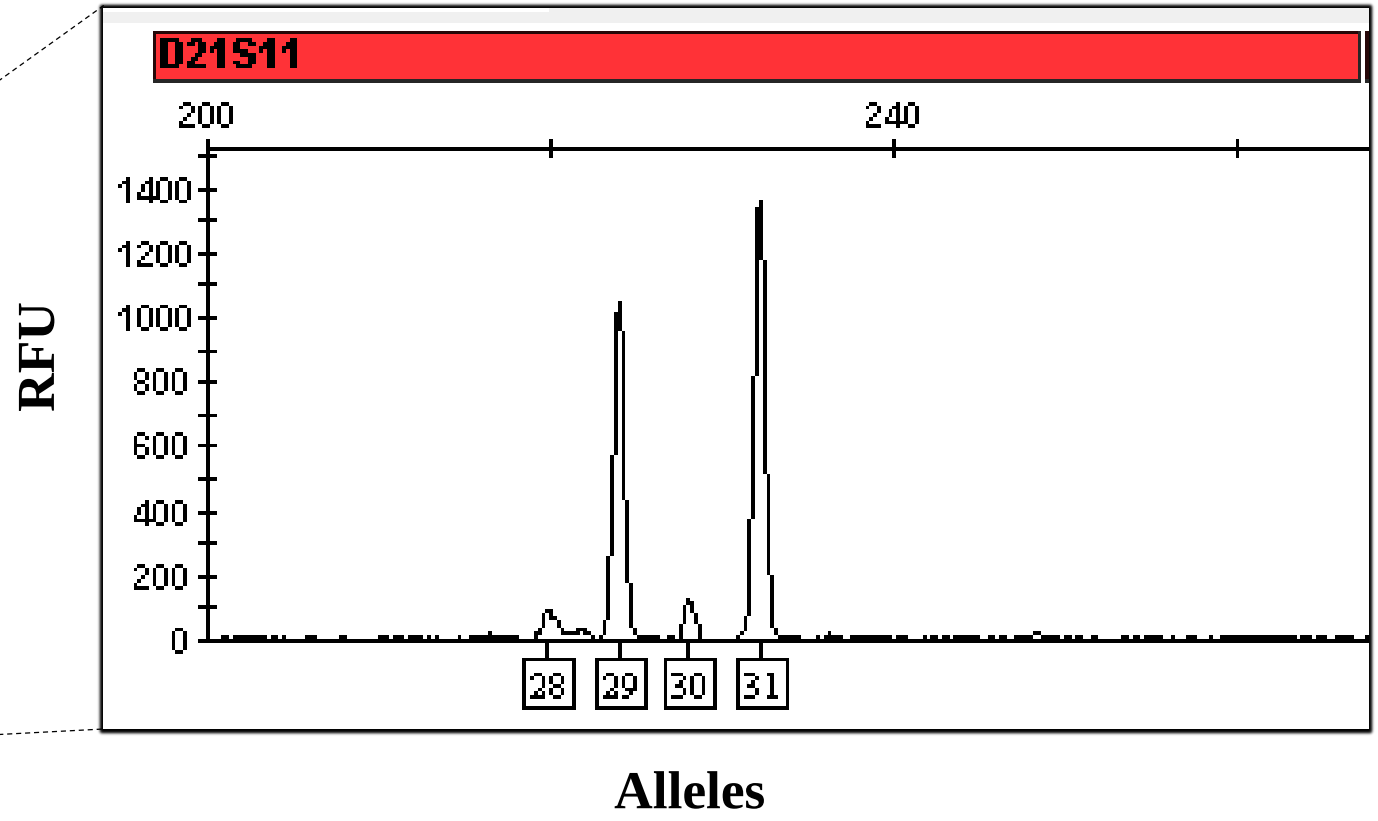
- NGS-STR profiles are susceptible to: **stochastic variation, signal noise, stutter artifacts, heterozygote imbalance,** and **allelic drop-out/in**

Comparison of conventional CE versus NGS-STR data analysis

Data Analysis by CE

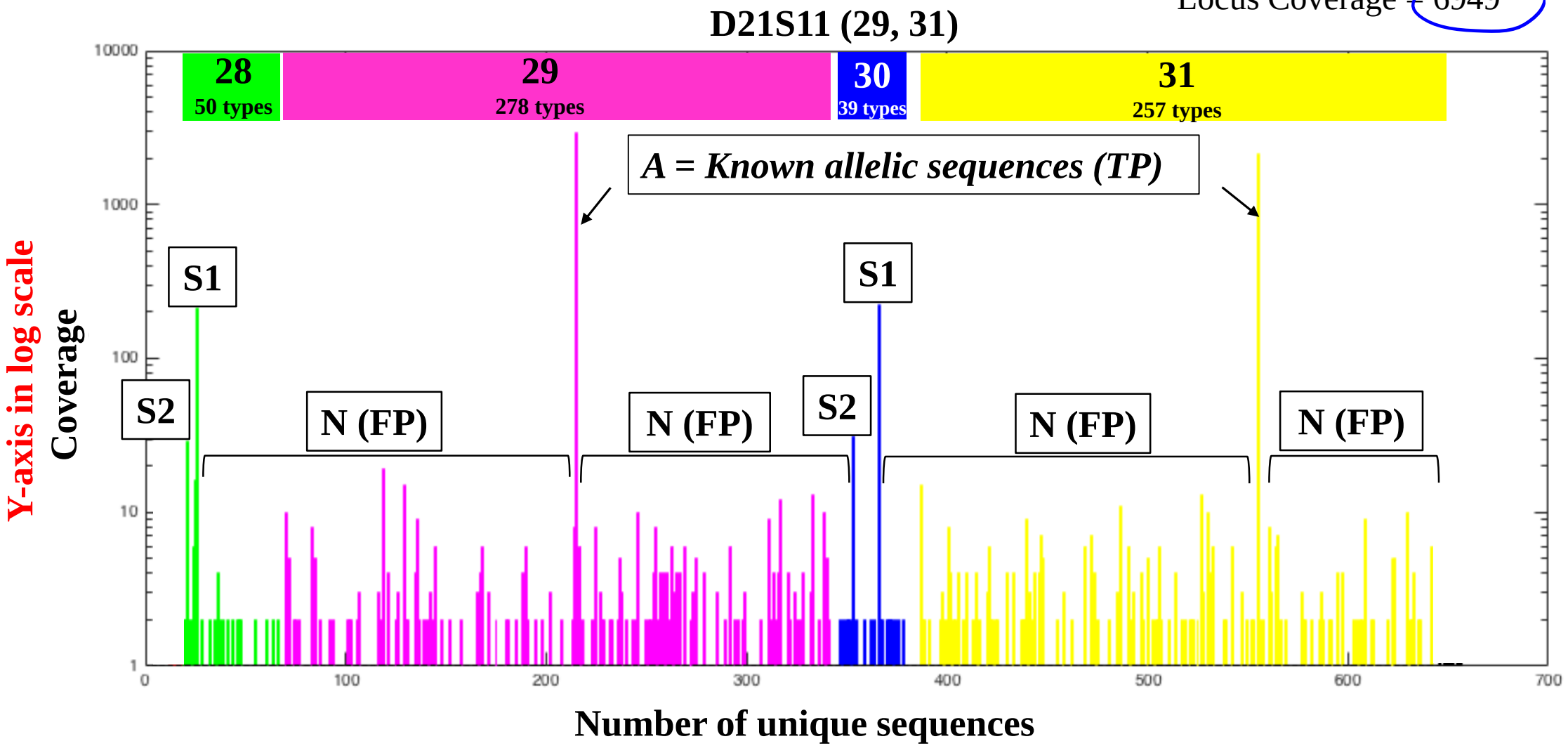


CE-D21S11



Data Analysis by NGS

Total number of unique sequences = 646
Locus Coverage = 6949



A = Known allele sequences (true positives: TP)

S1 = Back stutter of the LUS of the basic repeat motifs within an allelic sequence

S2 = Back stutter sequences not attributed to S1

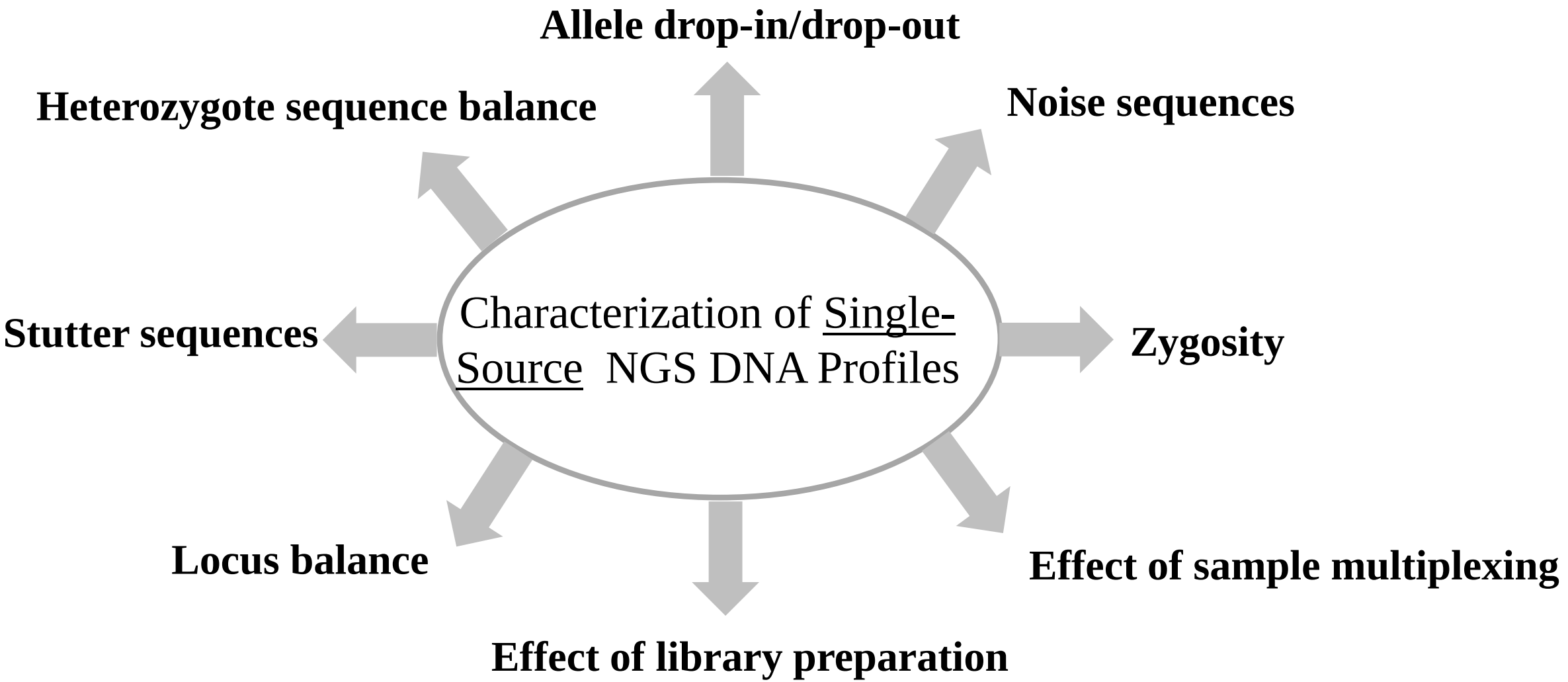
N = Noise sequences (false positives: FP)

What needs to be evaluated to:

Apply NGS-STR genotyping to forensic casework

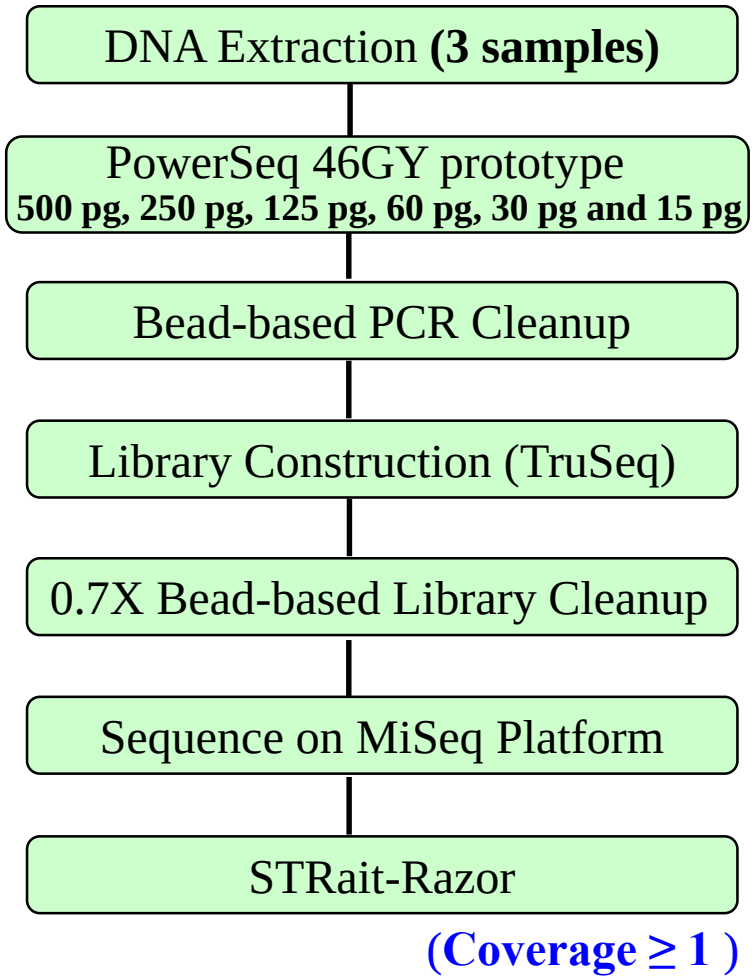
Establish probabilistic models for interpreting NGS-STR profiles?

We need to generate and characterize NGS-STR data



Sensitivity study of single-source DNA profiles generated by targeted sequencing

NGS sensitivity experimental design



PowerSeq 46GY System Prototype									
	1	2	3	4	5	6	7	8	9
A	500 pg	500 pg	500 pg	500 pg	500 pg	500 pg	500 pg	500 pg	500 pg
B	250 pg	250 pg	250 pg	250 pg	250 pg	250 pg	250 pg	250 pg	250 pg
C	125 pg	125 pg	125 pg	125 pg	125 pg	125 pg	125 pg	125 pg	125 pg
D	60 pg	60 pg	60 pg	60 pg	60 pg	60 pg	60 pg	60 pg	60 pg
E	30 pg	30 pg	30 pg	30 pg	30 pg	30 pg	30 pg	30 pg	30 pg
F	15 pg	15 pg	15 pg	15 pg	15 pg	15 pg	15 pg	15 pg	15 pg
G	**Sample A**			**Sample B**			**Sample C**		
NGS Workflow									

How should noise thresholds be set for NGS?

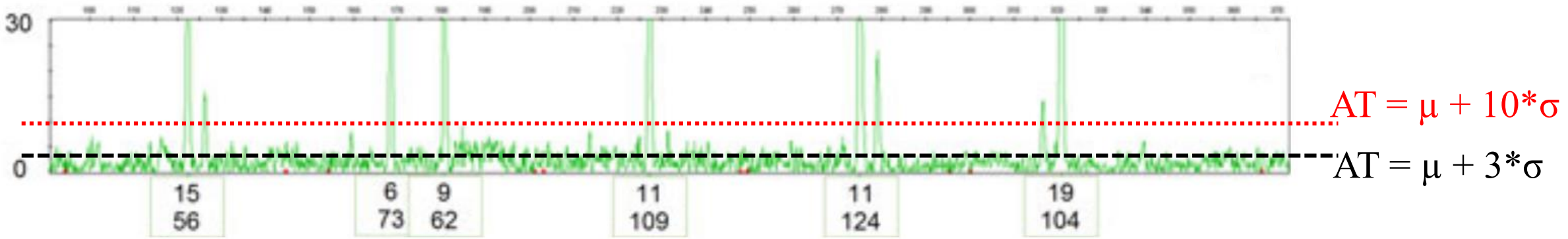
CE analytical threshold is most commonly determined by:

$$AT_{M1} = \bar{Y}_{bl} + ks_{bl}$$

- *k = Numerical factor (e.g. k=3)*

Average RFU signal

STDEV of the signal



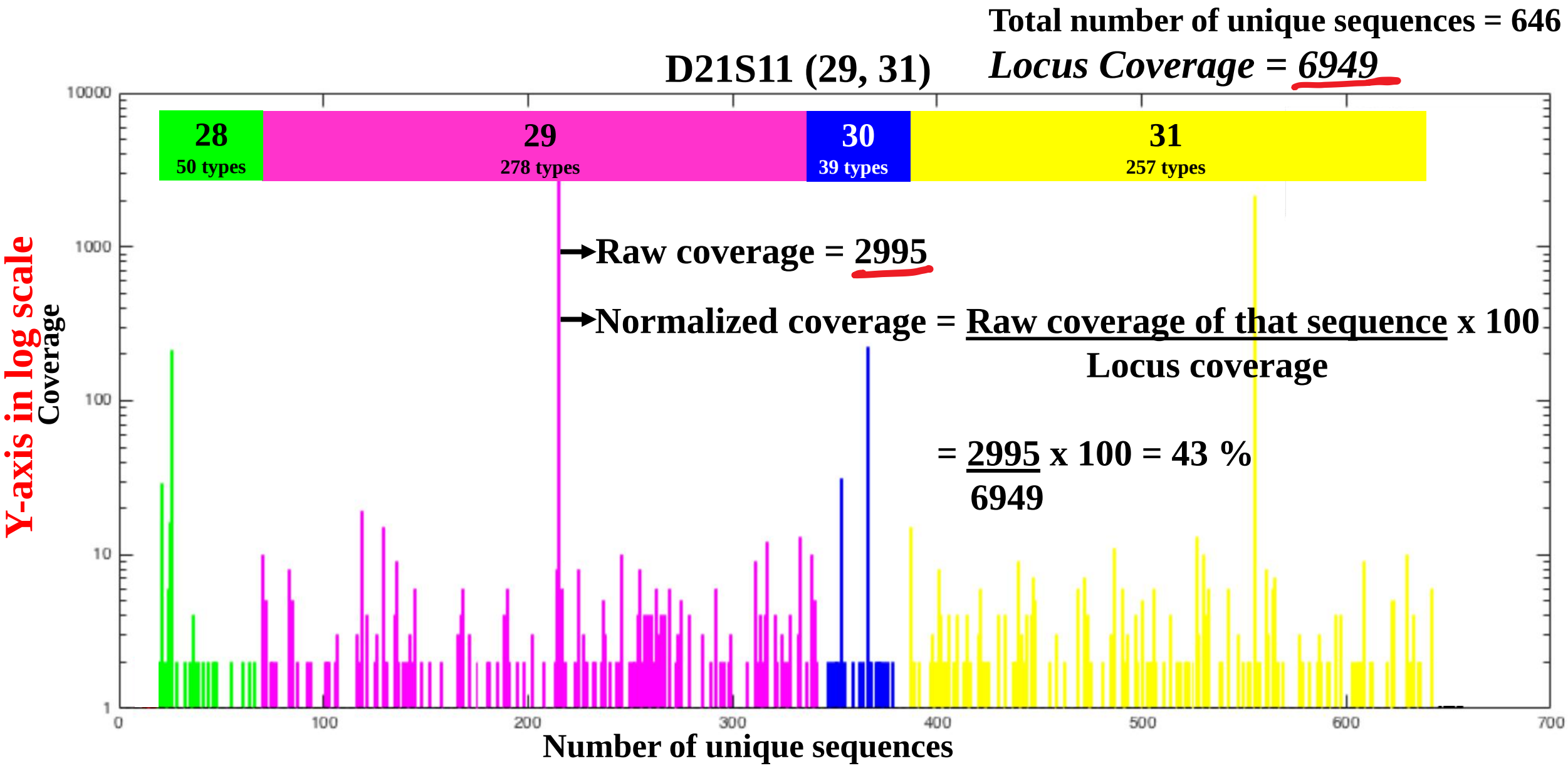
- **Dye-dependent**
- **Peak height (RFU)**

J. Bregu, D. Conklin, E. Coronado, M. Terrill, R.W. Cotton, C.M. Grgicak, Analytical thresholds and sensitivity: establishing RFU thresholds for forensic DNA analysis, Journal of forensic sciences 58(1) (2013) 120-9.

How to set noise thresholds for NGS?

CE-noise thresholds	NGS-noise thresholds
Peak Height (RFU)	Raw coverage or normalized coverage ?
$\mu + 10*\sigma$	?
Dye-dependent	▪ Protocol-dependent? ▪ DNA amount-dependent ? ▪ Locus-dependent ? ▪ DNA amount and locus-dependent ?

Raw coverage and normalized coverage



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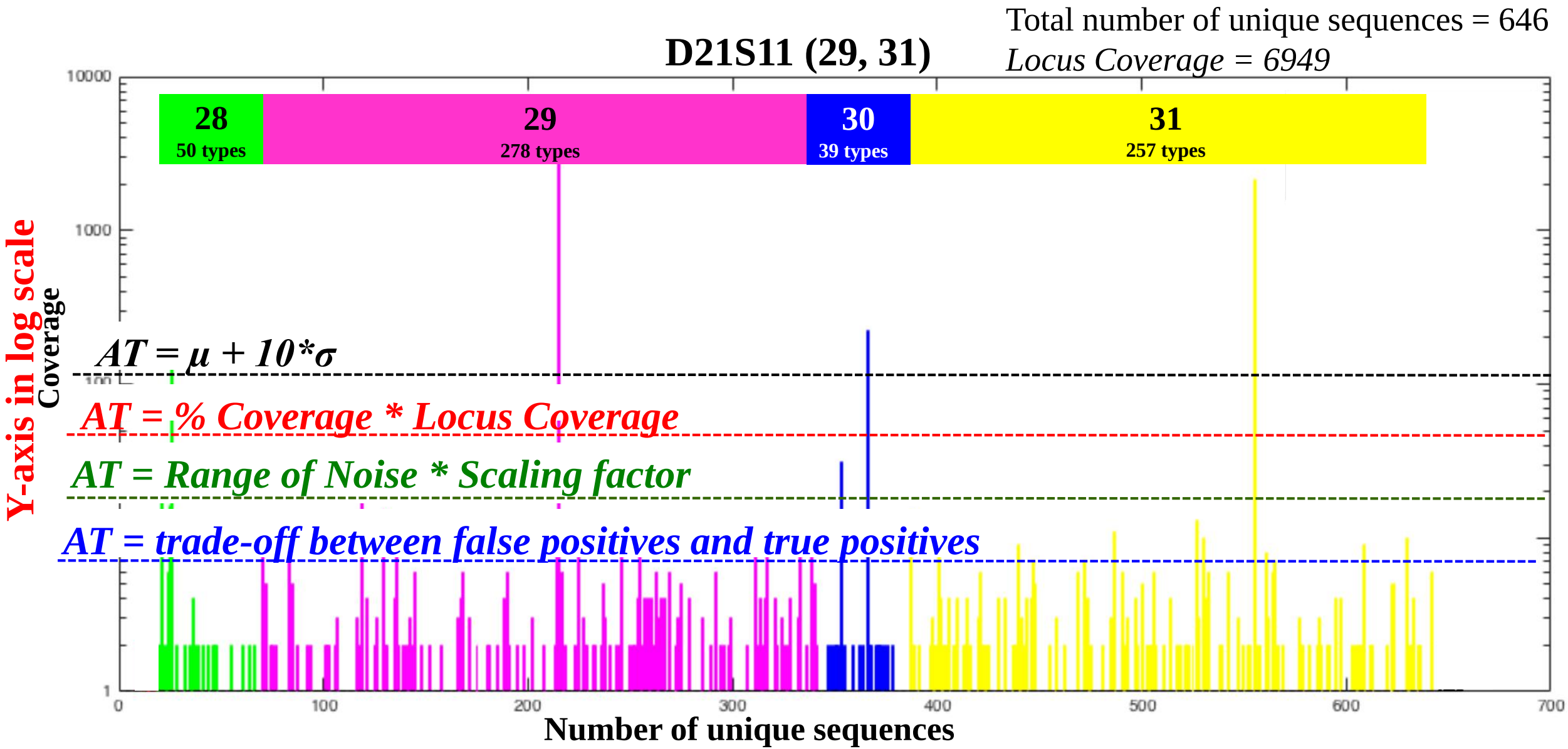
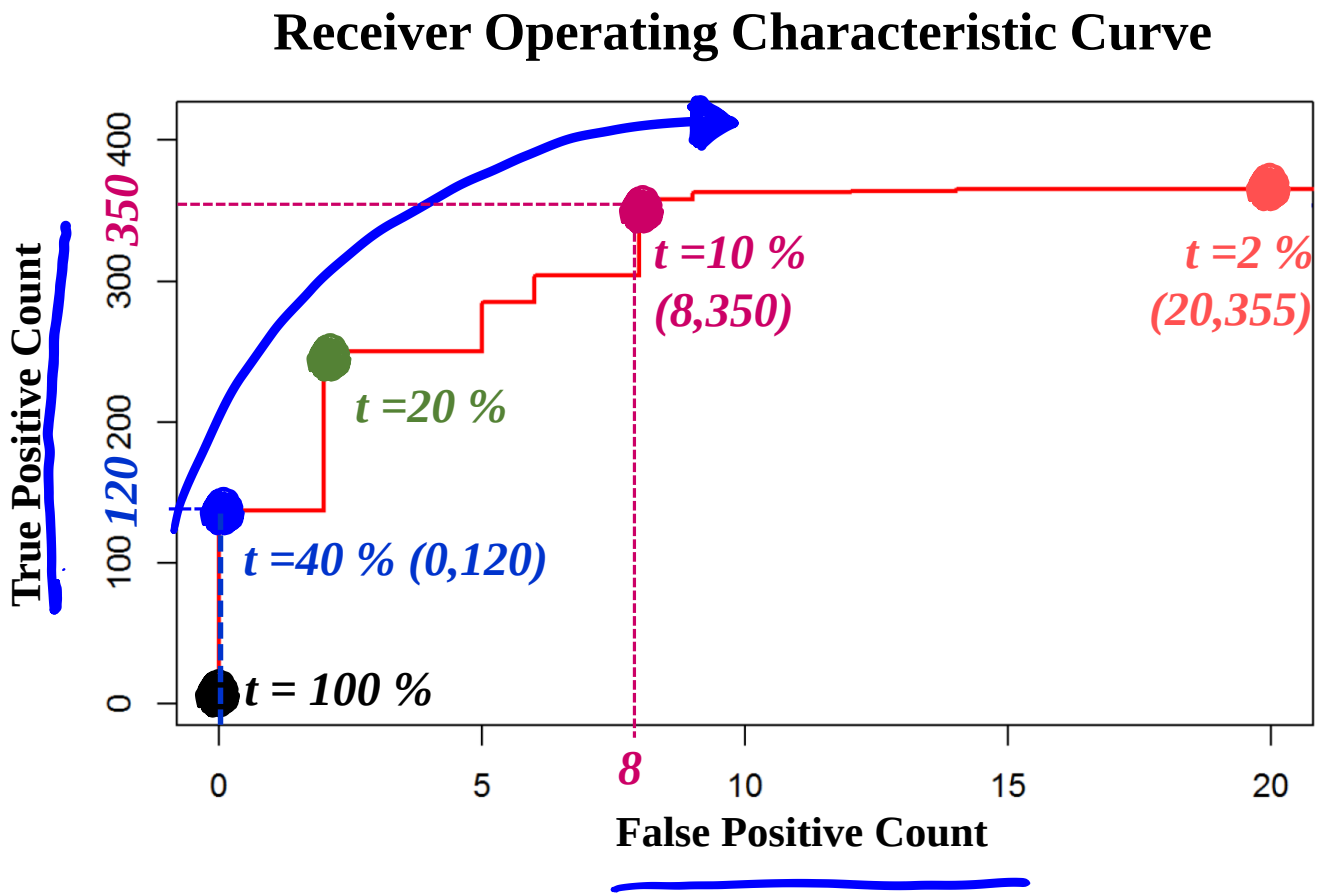


Evaluating the tradeoff between the allelic (true positives) and noise sequences (false positives) using Receiver Operating Characteristic (ROC) Curves

Receiver Operating Characteristic (ROC) Curve

- **True positive count on y-axis**
  - Count of known allelic sequence
- **False positive count on x-axis**
  - Count of noise sequences
- **Captures all the noise thresholds (t) simultaneously**
- **High noise threshold**
  - Decrease in detecting known alleles
  - Decrease in drop-in
- **Low noise threshold**
  - Increase in detecting known alleles
  - Fewer drop-outs
  - Increase in drop-in



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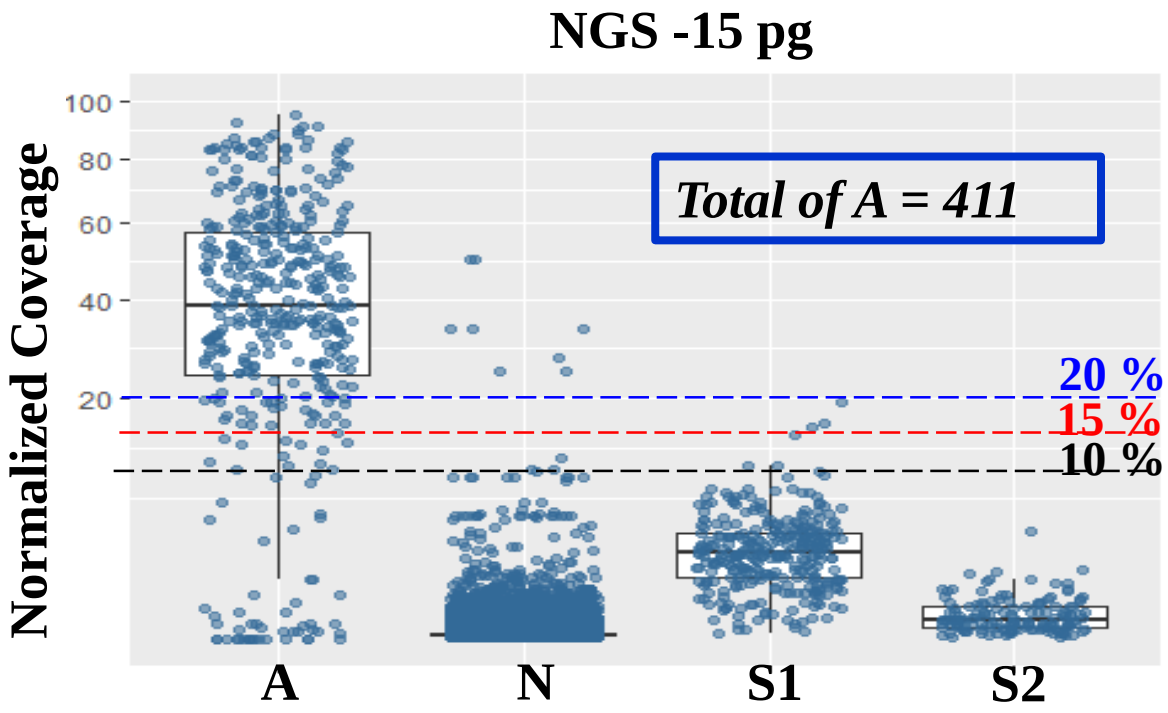
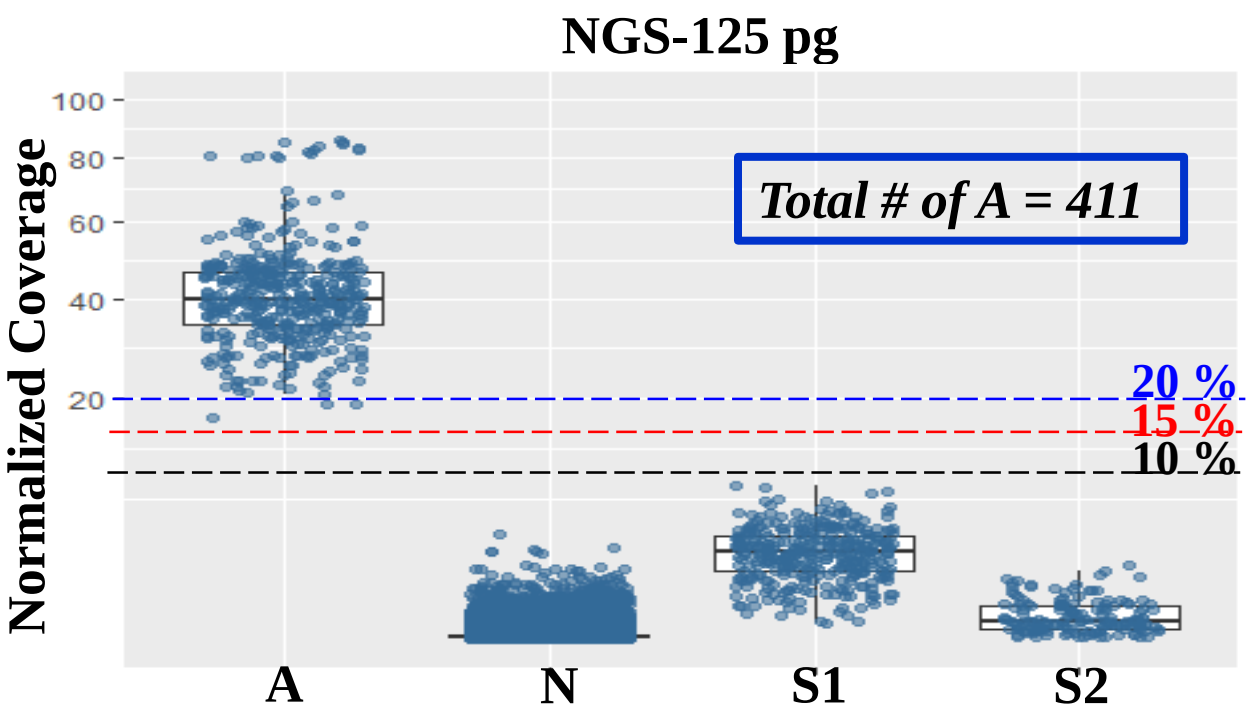
Impact of DNA Template Amount on the Distribution of Known Allele, Stutter, and Noise Sequences





t	A	DO	N	S1	S2
10 %	363	48	11	6	0
15 %	350	61	8	3	0
20 %	327	84	8	0	0

t	A	DO	N	S1	S2
10 %	411	0	0	0	0
15 %	411	0	0	0	0
20 %	408	3	0	0	0

- As expected, improved discrimination between known alleles (A) and the remainder of the sequences (N, S1, and S2) is observed as the amount of DNA template increases.
- Values of 10 %, 15 %, and 20 % are ONLY used for illustrative purposes and not as recommended thresholds. Each lab should perform sensitivity experiments and establish a threshold for interpretational purposes.

Future work

- Generate and characterize a large number of single-source samples with varying quantity and quality
- Characterize stutter sequences
- Characterize noise sequences
- Evaluate and compare using raw coverage and normalized coverage noise thresholds
- Evaluate DNA amount- and/or locus-dependent noise thresholds
- Generate and analyze mixture samples for performance check

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Contact: sarah.riman@nist.gov